dependent on new claims 75 and 83. New claims 93 and 94 are based on previous claim 70, but with additional language to clarify the method claimed. New claims 96 and 97 are based on previous claim 56 together with the passage on page 29 of the description.

Before addressing the new claims and the references in detail, the following general remarks are offered in order to set the present invention in context.

By the priority date of the present invention (23rd January, 1986), it was well known to transform bacterial or eukaryotic host cells with a vector containing a gene which endowed the host cell with a recognisable phenotype. For instance, in bacterial systems, host cells which are susceptible to various antibiotics were used. These host cells were then transformed with a vector which included on it a gene endowing transformant host cells with the ability to withstand a particular antibiotic. The host cells were then grown in a medium containing the particular antibiotic. Cells which had not been transformed succumbed to the effect of the antibiotic, leaving only transformant cells growing in the medium. Thus the gene for antibiotic resistance acted as a selectable marker.

In bacterial systems, there was a wide choice of host cells available which are susceptible to various antibiotics.

Each of these host calls completely lacks a gene endowing the cells with resistance to the antibiotic. Vectors containing genes which endow cells with resistance to the various antibiotics were also available. Thus, it was readily possible to design an experiment to transform a bacterial cell and to identify transformant cells using an antibiotic resistance gene as a selectable marker.

Such antibiotic resistance genes may also be used as selectable markers in eukaryotic host cells as these calls normally lack genes which code for antibiotic resistance. However, these antibiotic resistance genes are not susceptible to amplification in eukaryotic cells. This is a disadvantage of antibiotic resistance genes, as amplifiable selectable markers are often required in eukaryotic systems to co-amplify desired genes to obtain satisfactory expression levels.

Thus, alternative systems have been evolved for use with eukaryotic cells, for instance as shown by the Ringold and Axel citations (US-A-4 656 134 and US-A-4 399 216). In eukaryotic systems, host cells are selected which lack a gene essential for enabling the cell to grow on a selected medium. Axel et al. refer to host cells which lack the gene for thymidine kinase (tk) and Ringold refers to cells which lack the gene for dihydrofolate reductase (dhfr). tk^- cells

do not grow on HAT medium, and dhfr$^-$ cells are unable to grow without nucleosides. Such eukaryotic cells can be transformed with vectors containing tk or dhfr genes. Transformants containing the tk gene can survive in HAT medium and those containing the dhfr gene can survive in a medium lacking nucleosides. Thus, the tk and dhfr genes can act as selectable markers in those particular cell types.

Axel et al. also show a variation an this theme. dhfr is inhibited by the drug methotrexate (MTX). Thus, even cells which are dhfr$^+$ cannot grow in a medium containing MTX. Axel et al. show that there is available a mutant dhfr gene. The mutant dhfr produced by this gene is much less susceptible to the effects of MTX than is wild-type dhfr. Thus, dhfr$^+$ cells can be transformed with the mutant dhfr gene and then grown in medium containing MTX. Those cells which contain the mutant dhfr gene will survive in the medium. Thus, the mutant dhfr gene can act as a selectable marker in this system.

By analogy with the work carried out in bacteria, it was the conventional wisdom that a gene could only be used as an effective selectable marker if that gene was entirely absent from or non-functional in the host cell to be transformed. It was believed that, if the gene was active in the call to be transformed, it would not be possible effectively to

select transformant cells from non-transformant cells on the basis of the phenotype endowed by the gene. This was because it was believed that the effect of the endogenous gene would provide the same phenotype as that provided by the introduced gene.

The main exception to this was the use of the mutant dhfr gene as shown by Axel, but this in effect worked on the same principle because, in media containing MTX, the wild-type dhfr gene was ineffective and the $dhfr^+$ cell was mutant $dhfr^-$.

As alluded to above, another use for selectable markers, as set out for instance in the Ringold and Axel et al. was in order to carry out co-amplification processes. It had been discovered that it was possible to transform a host cell with two DNA sequences, either linked or unlinked, and then to culture transformant cells under such conditions that one of the two DNA sequences spontaneously amplified. As long as the sequence which amplified comprised a gene for a selectable marker, it was possible to select those cells in which spontaneous amplification had taken place. What had also been discovered was that when the first DNA sequence amplified, the second sequence also amplified.

Again, the conventional wisdom was that co-amplification processes could only be carried out efficiently either in

host cells lacking the gene encoding the selectable marker used for selection or in cells containing an inhibitable active endogenous gene and using a resistant mutant gene as the selectable marker. It was reasoned that if an active or uninhibited endogenous gene was present, this would also spontaneously amplify under the culture conditions, and thus it would be impossible to select cells in which the endogenous gene was amplified from cells in which the exogenous gene was amplified. Thus, co-amplification processes were carried out in the same sort of cells as were used for selectable marker processes. Generally, the system used for co-amplification involved transforming $dhfr^-$ CHO cells or $dhfr^+$ cells with a first DNA sequence encoding either a dhfr gene or a mutant dhfr gene respectively and a second DNA sequence encoding a gene for a desired protein. The cells were then cultured in a medium lacking nucleosides or containing MTX respectively. Surviving cells contained the exogenous dhfr gene or mutant dhfr gene respectively. The surviving cells were then cultured in media containing progressively increasing concentrations of MTX and cells in which the dhfr or mutant dhfr gene spontaneously amplified were selected. The whole basis for this co-amplification process was the fact that the original host cell line did <u>not</u> contain a dhfr or a mutant dhfr gene and so the selected

cells could only be transformants containing the exogenous gene.

The only exceptions to this were work by de Saint Vincent and Murray, but, as discussed below, their systems were not able usefully to effect amplification.

On the basis of this conventional wisdom, the skilled person would not have expected to be able to use an exogenous GS gene as a selectable marker in co-amplification processes in a host cell containing an active endogenous GS gene. The expectation would firstly have been that it would not have been possible to select transformed from untransformed cells. Secondly, it would have been expected that it would not have been possible to select amplified transformant cells from amplified untransformed cells, since it would have been expected that the endogenous gene would be as likely to amplify spontaneously as the exogenous gene.

In eukaryotic cells there were only a limited number of host cells which lacked an effective gene for an amplifiable selectable marker. Thus, the person skilled in the art had only a very limited choice of eukaryotic cell lines which could be used. This could be a significant disadvantage in many cases, for instance because the cell lines were not suitable for the use intended or were only slow growing.

At the priority date of the present invention, the main cell lines used in amplifiable selectable marker experiments were dhfr$^-$ CHO cells (referred to in Ringold et al.) and, less commonly, Ltk$^-$ mouse cells (referred to in the Axel et al.). There were other cell lines available as well, but the most commonly available cell line was the dhfr$^-$ cell line. In this respect, reference is made to the discussion on page 2, line 1 to page 3, line 24 of the present application.

Thus, at the priority date, there was a need to identify another gene which could be used as an amplifiable selectable marker. In accordance with conventional wisdom, and despite the work of de Saint Vincent and Murray (discussed below), those skilled in the art were still looking for eukaryotic cell lines which lacked a particular gene or for resistant mutants of endogenous inhibitable genes so that the cell line would be susceptible to transformation with a gene to endow transformant cells with a selectable phenotype. The main thrust of the search was towards cell lines lacking the gene in question. It would not have occurred to the skilled person that a gene which was active in most host cells could be used as a selectable marker.

The applicants discovered surprisingly and unexpectedly that exogenous glutamine synthetase (GS) genes could be used

effectively as amplifiable selectable markers in host cells containing an active, endogenous GS gene. In particular, the present inventors discovered that transformant cells could be selected using only low levels of phosphinothricin or methionine sulphoximine (MSX) (inhibitors of GS), despite the fact that the host cell contains an active endogenous GS gene. They also discovered that a number of rounds of amplification could be carried out to give an about 100-fold increase in copy number, yet still maintain tolerable levels of MSX. This was entirely contrary to conventional wisdom and was very unexpected in the light of the work by de Saint Vincent and Murray (discussed below). This is the basis for the present invention.

As regards amplification, the applicants discovered surprisingly and unexpectedly that it is possible to use an exogenous GS gene as a selectable marker in a co-amplification process using host cell containing an active endogenous GS gene. The applicants discovered that the exogenous GS gene (and any other genes co-transformed with it) spontaneously amplified at rates significantly greater than the rate at which the endogenous gene spontaneously amplified. Thus, not only could the exogenous GS gene be used as a selectable marker, but also it could be used in co-amplifica-

tion experiments, even in host cells containing an endogenous GS gene. This is a further basis for the present invention.

There were two attempts made to use native genes as selectable markers in cells which already contained an active endogenous gene. In the first case, de Saint Vincent et al. used a gene encoding CAD, a multifunctional enzyme, to transform host cells containing an active endogenous CAD gene. Their work is reported in Cell, 27, 267-277, 1981, a copy of which will be submitted for the Examiner's convenience. The system used by de Saint Vincent at al. involved the cloning of a functional CAD gene into a cosmid vector to produce vectors of over 40 kb in length. Such vectors are very unwieldy and could not readily be used for cotransformation in commercial systems. The cosmid vectors were introduced by protoplast function into wild-type CHO cells. It was found to be possible, by using highly elevated concentrations of PALA (a specific inhibitor of the CAD enzyme) to select transformed cells containing an amplified number of donated CAD genes. This system is clearly very cumbersome and cannot readily be used to provide a selectable marker in CAD^+ cells. Moreover, since selection requires that the donated CAD gene be amplified, it is not possible to use this system for further amplification. Thus, the

system disclosed by de saint Vincent et al. is not in any way a practical proposal.

The other attempt was made by Murray et al. and is reported in Mol. Cell. Biol., 3, 1, 32-43, 1983. A copy of Murray et al. will be submitted for the Examiner's convenience. It relates to further work using a wild-type dhfr gene. The wild-type dhfr gene was put in a vector under the control of a very strong promoter (HSV-ltr promoter). The vector was used to transform $dhfr^+$ cells. It was found that, by use of very high levels of MTX, it was possible to select for cells containing the donated dhfr gene. However, the levels of MTX which were required made it impossible to amplify the dhfr gene to any reasonable degree. Moreover, since the dhfr gene is under the control of such a strong promoter, it is likely that any other gene cotransformed with the dhfr gene would only be poorly expressed, even if amplification could be achieved. It can thus again be seen that this highly specialised system would not be of any use in a commercial situation.

As far as the applicants are aware, neither the de Saint Vincent nor the Murray work has been followed up. They remain as academic curiosities. Commercial work and most experimental work in this area concentrated on the $dhfr^-$ cell line or mutant dhfr gene work described above.

Applicants respectfully point out the use of a GS gene as a selectable marker and in co-amplification processes is highly advantageous. In particular, it increases many fold the number of host cells that can be used in such processes. As is stated in the specification at page 5, line 23 to page 6 line 1, active endogenous GS genes are present in a wide variety of cells. It would previously have been considered that these cells would not have been suitable as host cells if an exogenous GS gene were to be used as an amplifiable selectable marker. However, the applicants have shown that all these cells (assuming they are suitable in other respects) can be used for this purpose. Thus, the skilled person's choice of cell line has been dramatically increased.

The use of an exogenous GS gene as a selectable marker also has advantages over the dhfr system. In the dhfr system, the selective agent is MTX, a highly toxic substance, which must he used at concentrations which are well above safe levels for operators using the system. Using GS, it is possible to select for transformants using a GS inhibitor. In the preferred method, MSX is used as the selective agent. MSX is much less toxic than MTX and is used at concentrations which are much less toxic. Thus, the use of a GS-based system is considerably safer than the use of a dhfr based system. Moreover, it is possible to further

reduce the level of MSX used by adding methionine to the medium. Methionine potentiates the effect of the MSX. There is no way in which the effect of MTX can be potentiated. Thus, the GS-based system has many advantages over dhfr-based systems.

It can thus be seen that the present invention is based on the discoveries that, contrary to conventional wisdom, an exogenous GS gene can be used not only as a selectable marker but also in co-amplification processes in cells containing an active endogenous GS gene. These discoveries are surprising and unexpected and lead to systems having distinct advantages over prior art systems.

The specification and previous claims 62, 64-69 and 72-74 were rejected under 35 USC 112, first paragraph. Applicants respectfully traverse the rejections. Applicants respectfully point out that, at the time the present invention was made, it was well known that co-amplification could be carried out by use of either linked or unlinked DNA sequences. That this was well known is amply illustrated merely by reference to Axel et al. This is in particular directed to the co-amplification of unlinked DNA sequences. Following the disclosure by Axel, and well before 1986, co-transformation using unlinked DNA sequences, especially in dhfr$^-$ cells, had become a well-known technique which any

person skilled in the art could carry out. Thus, it would not require any undue experimentation to put this technique into effect using a GS gene. The invention lies in the discovery that a GS gene could be used in this technique, not in the technique itself. The rejections are therefore respectfully traversed.

The Examiner claims 51, 53 to 55, 62 to 69, and 72 to 74 under 35 USC 112, first paragraph. Applicants respectfully traverse these rejections and submit that in raising these rejections, the Examiner appears to be taking no account of the ordinary knowledge of the person skilled in the art. At the priority date of the present application it was well known that certain vectors were suitable for use in some, but not other, host calls. However, it was also well known that for any host cell a suitable vector was available or could readily be produced. In many instances "cassette" vectors were available for particular cell lines. Such cassette vectors include all the necessary features to enable the vector to operate in the particular cell line. The vector also contains a unique restriction enzyme site. By cutting the vector at this site, a desired DNA sequence, containing one or more genes, can be inserted between the cleaved ends in such a way that the inserted gene(s) will be expressed once the cell is transformed with the vector.

Thus in the present came, a skilled person would be able readily to insert a GS gene and, if desired, other genes into a suitable vector for use in a selected cell line. Again, the invention lies in the use of the GS gene, not in the particular vector in which the GS gene is located, although, as the application shows, some vectors have additional advantages over others).

It is no doubt true that a vector designed for use in a CHO cell probably would not be suitable for use in a yeast or a bacterial cell. However, this is vary well known to the person skilled in the art. Therefore, if such a person wished to operate the present invention in yeast rather than CHO cells, all that he would have to do would be to excise the appropriate DNA sequences from the CHO-type vector and insert it into an yeast type vector. This would not present the skilled person with any problem which could not be solved only by use of his ordinary skills

Much the same arguments are true as regards promoters. The skilled person is well aware that certain promoters can only be used in certain cells, whereas other promoters are usable in other cells. If the skilled person decided to use a particular sort of host cell, then as a matter of course a promoter which operated in that host cell would be selected.

The skilled person would not select a combination of promoter and host cell he knew would not work.

As regards the mechanism of amplification, it is pointed out that amplification is not a process which in controlled by mechanisms inserted into the host cell by the operator. Amplification is a spontaneous event common to most, if not all, cell types. In carrying out amplification processes, all that is happening is that the skilled person is using the selectable marker to select for transformant cells in which spontaneous amplification has taken place. Thus, the skilled person does not need to provide any special mechanism to be inserted into the host call. All he needs to do is to provide a host cell in which spontaneous amplification is known to occur.

It is thus believed that, if the ordinary knowledge of the person skilled in the art is taken into account, the description is enabling to the full extent of the present claims. Thus, the 35 USC 112, first paragraph rejections to previous claims 51, 53 to 55, 62 to 69, and 72 to 74 are respectfully traversed.

The rejections under 35 USC 112, second paragraph of previous claims 44, 48, 49 and 69 are respectfully traversed in view of the amended new claims.

The rejections of claims 39 to 74 under 35, USC 103 (in the Office Action at page 5, first paragraph to page 8, fifth paragraph) are respectfully traversed in view of the claims 75 to 97. It is respectfully submitted that the amended new claims are not at all obvious in view of Sanders et al., Ringold, Axel et al., Pennica et al., and Young and Ringold, for the reasons set out below.

Sanders is an academic paper relating to an investigation of the GS gene in CHO cells. Sanders shows that the genomic GS gene can be amplified by exposing CHO cells to increasing levels of MSX. Sanders also reports on the partial cloning of the CHO GS gene. However, Sanders does not show the cloning of the complete GS gene, nor does it in any way relate to the use of the GS gene as a selectacle marker or in co-amplification processes.

In particular, applicants respectfully believe that the Sanders paper points away from the use of a GS gene in these processes. The fact that the endogenous GS gene in CHO cells can be amplified in its natural chromosonal environment would lead a skilled person to expect that amplification of the endogenous gene would prevent selection of transformants containing amplified exogenous GS genes. In this respect, reference is made to the general remarks made above.

It is respectfully submitted that the Examiner is applying hindsight reconstruction in order to try to show that any of the claims in the application relating to selectable markers and co-amplification are obvious in view of Sanders. Applicants respectfully believe that a skilled person could not gain any incentive from Sanders even to consider using a GS gene as a tool in recombinant DNA technology, let alone in the specific methods now claimed.

Pennica relates solely to the production of tissue plasminogen activator (tPA) by recombinant DNA technology. Pennica has nothing at all to do with the use of selectable markers or co-amplification. The only relevance it has to the present claims is to show that a recombinant DNA sequence encoding tPA was known. Thus, it merely shows that new claims 82 and 90 are enabled, even if there were no specific teaching in the description relating to the production of tPA.

Ringold is concerned with amplification of DNA sequences. However, applicants respectfully believe that Ringold points away from, not towards, the present invention. As can be seen immediately from the abstract of Ringold, it relates to the use of genes which can complement auxotrophic, not prototrophic, host cells. This theme is continued throughout Ringold. Reference is made to the use of dhfr,

CAD, metallothionein, asparagine synthetase and drug resistance genes for use in auxotrophic hosts. Thus, applicants respectfully believe that there is nothing in Ringold to suggest to the skilled person that a gene which is normally present in host cells could be used as a selectable marker or in amplification processes. Therefore, a skilled person reading Ringold would not even contemplate using an exogenous GS gene in an amplification process in a GS prototroph host cell.

Moreover, using an exogenous GS gene in an amplification process using a host cell containing an active endogenous GS gene would go entirely against the teaching of Ringold. It is an essential feature of the disclosure in Ringold that the host cell should be auxotrophic. A host cell containing an active endogenous GS gene would not be auxotrophic for glutamine.

Thus, even if a person looking at the Ringold citation also looked at the Sanders citation, applicants respectfully believe that he would have no reason to combine them. It is therefore submitted that the present claims are not at all obvious over Ringold, whether or not combined with Sanders.

Axel et al. is very similar to the Ringold citation in that it relates to amplification. However, Axel et al. primarily relates to amplification of unlinked DNA sequences.

Axel et al. and Ringold have in common that some of the host cells used are auxotrophic, that is they lack a gene vital for their survival in a selective medium. Axel et al. exemplifies the use of tk^- cells. For the same reasons as are set out above in relation to Ringold, applicants respectfully believe that there is nothing in Axel et al. to lead a skilled person to try using a wild-type GS gene as a selectable marker or in an amplification process. Certainly there is nothing to suggest that a GS gene could be used effectively as a selectable marker in a host cell containing an active endogenous GS gene. The only system shown by Axel et al. which uses a host cell containing an active gene is the system which uses the mutant dhfr gene. However, as set out above, this in effect is a system in which the gene which is used as the selectable marker is absent from the host call. It is therefore submitted that the new amended claims 75 to 97 are not at all obvious over Axel et al., alone or in any logical combination with any of the other references.

Donn et al. relates to the amplification of the endogenous GS genes in alfalfa cells and to the isolation of partial cDNA clones for the GS genes. The recombinant DNA work reported in Donn et al. was carried out to enable quantitative assessments of the degree of amplification. There

is no suggestion that the GS genes could, let alone should, be used as tools in recombinant DNA technology. It is therefore believed that the Donn et al. goes no further than Sanders et al. For the reasons set forth in relation to Sanders et al., it is respectfully submitted that Donn et al. does not render the present claims obvious, whether taken alone or in any logical combination with any of the other references.

Young is very similar to Sanders et al. It also shows the isolation of a cell line (in the Young case a mouse 3T6 cell line) which is resistant to high levels of the GS inhibitor MSX. Young also reports on investigations into the mRNA produced by the resistant cell line with a view to producing cDNA so that molecular cloning of the GS gene can be carried out. Like Sanders et al. and Donn et al., there is no suggestion at all that the GS gene, if successfully cloned, could, let alone should, be used as a tool in recombinant DNA technology. Thus, it is believed that Young does not render the new amended claims obvious, whether taken alone or in any logical combination with any of the other citations.

Applicants respectfully point out that a co-author of Young is Gordon M. Ringold, who appears to be the same Gordon M. Ringold who is named as the inventor in Ringold.

Ringold was granted on a continuation application filed on 12th April, 1985. This is over 18 months after the publication of Young. The Examiner appears to be contending that it is obvious that a GS gene could be used in place of the various genes mentioned in Ringold. If this is the case, it should have been obvious to Ringold in 1985. However, despite the fact that Ringold clearly knew about the GS gene, no mention of it appears in Ringold. Thus, applicants respectfully believe that this is a strong indication that the present claims are not at all obvious.

Applicants respectfully believe that none of the other references cited in the present application is more pertinent than the references discussed above. They are therefore not discussed here.

For the reasons set out above, the rejections under 35 USC 103 are respectfully traversed.

In view of the above amendments, and for the reasons discussed, applicants respectfully submit that their application is now in condition for allowance and such notice is respectfully requested.

Respectfully submitted,

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